ASSAY OF THE ACTIVATION STATE OF PLATELETS

FIELD OF THE INVENTION

All biomedical devices that recirculate blood or are inserted into the blood stream have a thromboembolic potential with attendant risk of cardioembolism. Various medical conditions also result in alteration of thrombotic potential. The present invention provides for quantitative measurement of platelet activation state. In particular, the invention relates to a platelet activation assay that uses modified prothrombin, which is activated by prothrombinase and which generates thrombin that does not activate platelets but can still be measured.

BACKGROUND OF THE INVENTION

Platelets are a major contributor to the pathology of thrombosis, particularly arterial thrombosis, where they form the bulk of thromboembolic occlusions. It is not clear to what extent such platelets are activated *in vivo* by the ordinary hemostatic pathway, initiated by exposure of the subendothelium, and to what extent by other causes, such as exposure to shear stress (Folie and McIntire, Biophys. J., 56:1121-1141, 1989; Slack *et al.*, Thromb. Haemost., 70:129-134, 1993).

Standard accepted clinical practice does not currently include the measurement of platelet activation state in patients at risk of thromboembolic disease or in patients undergoing anti-platelet therapy, such as low-dose aspirin treatment. It is well accepted that anti-platelet therapy very significantly reduces the risk of thromboembolic disease, and conversely that various agents likely raise the risk of such disease through a primary action on platelets. It is also

clear from studies of patients with prosthetic devices such as heart valves that in this case the greatly increased risk of development of thromboemboli is caused by platelet activation due to the device. The measurement of platelet aggregation in response to various agonist compounds is currently the norm for clinical studies of platelet defects, but for the most part this test is used to examine defects that cause bleeding tendencies rather than possible causes

of thrombotic disease. Further, the test measures a final event in platelet activation, and does not report the initial activity state of the platelets. Other, currently available tests which evaluate a patient's thrombotic potential include measurement of antithrombin III and proteins C and S in the blood, and PCR testing for factor V Leiden and a defect in the 3'-untranslated region of the prothrombin gene. For blood recirculating devices the feedback activation of the platelets by the thrombin produced also masks the flow induce effects of platelet activation by the device.

Platelet activation causes the exposure of two critical factors involved in the formation of thrombin: 1) anionic phospholipid–mainly phosphatidylserine–is transferred from the inner leaflet of the cell membrane to the outer leaflet, and there supports the binding and activation of the vitamin K-dependent proteins of coagulation: factors VII, IX, X, and prothrombin (see Jesty and Nemerson in Williams Hematology, 5th Edition, pp. 1227-1238, 1995 for a general review of the coagulation pathways); 2) factor V, which is present in the α-granules of the platelets, is concomitantly activated and the resulting factor Va, which is a required cofactor for prothrombin activation by factor Xa, is expressed on the membrane surface. Thus, activated platelets provide both of the major cofactors required for prothrombin activation by factor Xa. The complete complex, factor Xa + Va + anionic phospholipid, has been called the prothrombinase complex. A number of investigators, led by Rosing and Zwaal and their colleagues (Rosing *et al.*, Blood, 65:613-620, 1985; Bevers *et al.*, Blood Reviews, 5:146-154, 1991), have taken advantage of the platelets' providing these activities in using the kinetics of prothrombin activation in the presence of platelets as a measure of their "procoagulant" activity, and by extension of their activation state. However, the use of this assay is problematic because the enzyme generated and measured in the assay–thrombin–is a potent platelet activator. As the reaction progresses, the thrombin which is produced further activates the platelets and the thrombin generation rate increases. Thrombin generation which is measured in these assays is often non-linear and therefore unreliable.

There remains a need in the art for a quantitative assay for platelet activation, and particularly for a fast, simple assay for thrombin production that does not cause further platelet activation through a feedback mechanism.

SUMMARY OF THE INVENTION

This invention advantageously provides methods and kits for detecting platelet activation, and more particularly for quantitating the level of platelet activation or the platelet activation state, *e.g.*, for evaluating the thromboembolic potential of subjects. This measurement is important in conjunction with biomedical devices that recirculate blood, and for monitoring the endogenous activation state of platelets directly obtained from patients.

Thus, in one embodiment, the invention provides a method for detecting the activation state of a platelet. The method comprises detecting a prothrombinase product, such as thrombin, generated by platelet-mediated catalysis of a modified prothrombinase substrate, such as prothrombin, wherein the product generated from the modified substrate cannot activate platelets. Thus, the detection method avoids feedback activation of the platelets, permitting an accurate assessment of the state of platelet activation.

Also provided is a kit for detecting the activation state of platelets. The kit comprises a modified prothrombinase substrate, wherein a prothrombinase product generated from the modified prothrombinase substrate cannot activate platelets, and an assay for the product generated from the modified prothrombinase substrate. Since an activator of prothrombin such as factor Xa must be added to the platelets to generate the prothrombinase complex, this can be included in the kit as well. The kit can be used to practice the methods of the invention.

In preferred embodiments of both aspects of the invention, modified prothrombin is acetylated and the assay for thrombin is an assay for an activity of thrombin. Preferably the assay for an activity of thrombin is an assay for amidolytic activity, *e.g.*, using a colorimetric substrate for thrombin such as a *p*-nitroanilide peptide substrate for thrombin.

DESCRIPTION OF THE DRAWINGS

FIGURE 1. Activation of normal prothrombin in the presence of platelets, unactivated or activated with calcium ionophore A23187.

FIGURE 2. Activation of acylated prothrombin in the presence of platelets, unactivated, or activated with calcium ionophore A23187.

FIGURE 3. Activation of normal prothrombin in the presence of platelets, unactivated, or thrombin-activated.

FIGURE 4. Activation of acylated prothrombin in the presence of platelets, unactivated, or thrombin-activated.

DETAILED DESCRIPTION OF THE INVENTION

The current invention fills an unmet need in the art: most tests which evaluate a patient's thrombotic risk measure the propensity of the blood to clot, whereas the current invention measures the activation state of platelets. Assessment of the activation state of a patient's platelets, e.g., for patients at high risk of myocardial infarction or stroke, and for patients subject to invasive procedures, is of significant value to physicians. Data obtained from the methods of the present invention would be an important factor in determining the best approach in managing or treating a patient with a thrombotic disease, i.e., drug regimen and/or surgical intervention and/or device implantation. All medical devices that either recirculate blood or are inserted into the vascular system cause platelet activation and therefore have thromboembolic potential with attendant risks of cardioembolism and stroke. Thus, establishing the patient platelet's procoagulant state prior to such procedures may benefit their clinical outcome. The present invention comprises a method by which a highly accurate assessment of the activation state of the platelets in a sample of blood may be performed.

The level of platelet activation is proportional to the prothrombinase activity associated therewith. In order to assess the activation level of platelets, the methods of the present invention can be used to measure platelet-associated prothrombinase activity. The level of prothrombinase activity can be evaluated by measuring the level of prothrombinase product produced by the prothrombinase reaction. However, the natural prothrombinase product, thrombin, provides a positive feedback signal to platelets such that thrombin itself increases the platelet-associated production of thrombin. This positive feedback mechanism confounds efforts to accurately measure the initial level of thrombin production by platelets.

The present invention offers an improvement over the prior art in the use of a modified prothrombinase substrate in these assays. The modified prothrombinase substrate

comprises prothrombin altered so as to reduce or eliminate the ability of the corresponding thrombin to activate platelets, preferably, while preserving proteolytic activity of the thrombin. Modification of prothrombin, *e.g.*, by acylation under specific conditions, still allows the molecule to be activated normally, and the proteolytic activity of the molecule can be preserved, but the acetylated thrombin that is generated does not activate platelets. This enables the quantitative measurement of the activation state of platelets in the absence of thrombin-mediated platelet activation.

In a specific embodiment, human prothrombin was acetylated to produce a modified prothrombin. Upon activation by platelet-bound prothrombinase, this prothrombin generated a form of thrombin that does not activate platelets but retains its amidolytic activity on a chromogenic peptide substrate. If normal prothrombin were to have been used in this assay, the thrombin that is generated activates the platelets in a feedback manner, accelerating the rate of thrombin generation, thereby preventing accurate measurement of the initial platelet procoagulant activity. Acetylation of prothrombin was carried out over a range of concentrations of sulfo-N-succinimidyl acetate (SNSA). Acetylation by 3 mM SNSA at room temperature for 30 minutes at pH 8.2 in the absence of metal ions produced a modified prothrombin that has < 0.1% clotting activity (by specific prothrombin clotting assay), but it is activated by factor Xa (in the presence of either artificially activated platelets or exogenously added factor Va + anionic phospholipid) to produce thrombin activity that is measurable with a chromogenic substrate. Because the feedback action on the platelets is blocked, the rate of thrombin generation is linear, allowing quantitative measurement of the initial platelet activation state.

Thus, the invention provides for accurate detection, and more importantly quantitation, of the activation state of platelets. As used herein, the term "detection" means to obtain either qualitative and quantitative information about platelet activation. In preferred embodiments of the invention, the platelets are isolated from blood and plasma prior to testing with modified prothrombin to avoid clotting or other undesirable reactions. Efficient methods for isolating platelets, such as by selective filtration, density centrifugation, immuno-absorption, cell sorting, other techniques may be used advantageously in the practice of the invention provided that such methods do not themselves activate the platelets, or that

the technique minimizes activation, or that activation by the technique can be accounted for in testing..

The methods and kits of this invention advantageously replace fluorescence activated cell sorting (FACS) analysis, which is the current standard for evaluating the activation state of platelets. Flow cytometry cost a great deal: capital outlay and maintenance costs both drive the cost of testing with such equipment to unreasonably high levels. The cost savings of this invention represents another significant advantage.

The term "platelet activation state" or "activation state of platelets" is used herein to refer to initiation of thromboembolic potential or activity, or both, in platelets. It may involve, *inter alia*, the two critical factors required for the formation of thrombin: transfer of anionic phospholipid – mainly phosphatidylserine – from the inner leaflet of the cell membrane to the outer leaflet; and concomitant activation of factor V to factor Va, which is a required cofactor for prothrombin activation by factor Xa, on the membrane surface. Thus, the platelet activation state may refer to the level of prothrombinase activity associated with a platelet or the level of thrombinase which is produced as a result of the prothrombinase catalysis of prothrombin.

Prothrombinase refers to any substance which cleaves prothrombin in a manner which converts the prothrombin to thrombin. In the practice of the invention, Factor Va produced by platelets combines with exogenous Factor Xa in the presence of appropriate anionic phospholipids to create the prothrombinase system. Factor Xa (or Factor X), refers to the well known coagulation factor, which can be obtained from any animal source, preferably a mammalian source, and most preferably human. Because these enzymes are highly conserved throughout evolution, any Factor X (or Xa) orthologue can be used. Because of its abundance, Factor X from food animals, especially cows, pigs, or chickens, is useful. Other sources of Factor X include, but are not limited to, mice, rats, and fish. Reptilian Factor Xa (or Factor Xa homologues), such as the tiger snake venom enzyme that catalyzes prothrombinase cleavage, can also be used. Any of the foregoing can be obtained from natural sources or by recombinant expression (which further provides for use of genetically engineered and modified Factor X or Factor Xa proteins).

As used herein, the terms "prothrombinase substrate" and "prothrombinase product" refer to the substrate and product involved in prothrombinase-mediated catalysis. In a specific embodiment, prothrombin is a prothrombinase substrate; hereinafter, when referring generally to the invention the term "prothrombin" can be taken to mean a prothrombinase substrate. Similarly, thrombin is a prothrombinase product, and, throughout the application is interchangeable with prothrombinase product unless specified otherwise.

"Modified prothrombin" is prothrombin that is capable of catalysis by prothrombinase, but which generates modified thrombin that does not activate platelets. The modified thrombin may retain some or all of its proteolytic activity. "Modified thrombin" is thrombin which has been modified such that it does not activate platelets. Thrombin that does not activate platelets need not have a complete lack of platelet activating activity, though, for purposes of the present invention, it has less than about 10% of this activity, preferably less than about 5%, more preferably less than about 1%, and most preferably less than about 0.1% of platelet activating activity of normal thrombin, *e.g.*, as demonstrated in a clotting activity assay. Modified prothrombin or modified thrombin may comprise prothrombin or thrombin which is chemically modified by the addition of an acyl group, an acetyl group, a succinyl group, a maleyl group, a polyethylene glycol group, an acetylated polyethylene glycol group, a pyridoxal 5'-phosphate group or a dichlorotriazinylaminofluorescinyl group or any combination thereof. In preferred embodiments, when the chemical modification is acetylation, the acetyl group is donated by sulfo-N-succinimidyl acetate. Modified prothrombin or modified thrombin may also comprise prothrombin or thrombin, respectively, which contains mutations. In preferred embodiments, when the modification of prothrombin is derived from a mutation, the prothrombin allele is *Metz* or *Quick I*. The *Quick I* allele is prothrombin including a mutation wherein arginine at amino acid position 382 is changed to a cysteine.

A "blood sample" is any portion of blood containing platelets for testing in accordance with the invention. Such a sample can be obtained from, in, or around a biomedical device that could be activating platelets, *e.g.*, through shear stresses. Alternatively, the blood can be drawn from blood vessels distal to the location of a biomedical device. In a specific embodiment, a device that involves removal and return of

blood, such as a pheresis or dialysis machine, can include a conduit to permit removal of a blood sample for testing in accordance with this invention. A blood sample may also be obtained directly from a subject by venipuncture.

Moreover, platelets obtained from blood, for example by platelet-pheresis, can be tested for their activation state or for their competence to be activated *in vitro* in accordance with this invention, so that upon return to the patient or a tissue matched recipient, the ability of the platelets to mediate thrombosis is established.

The methods of the present invention may be performed on blood from a subject or patient wherein the terms subject and patient refer to any animal whose blood comprises platelets. In preferred embodiments, the subject or patient is a human, however, the scope of the present invention includes other animals such as cows, pigs, mice, rats, birds, fish, snakes and others.

In a specific embodiment, the term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

Modified Prothrombin

Any modification that yields prothrombin that is a substrate for a prothrombinase enzyme complex can be used in the practice of the invention, provided that the thrombin generated from such modified prothrombin does not activate platelets. Thus, in addition to acylated, and particularly acetylated, prothrombin, the invention contemplates using naturally occurring or recombinantly generated prothrombin molecules that produce thrombin that does not activate platelets, such as the prothrombins Metz (Rabiet *et al.*, Blood, 63:927-934, 1984) and Quick I (Henriksen and Owen, J. Biol. Chem., 262:4664-4669, 1987; Henriksen and Mann, Biochemistry, 27:9160-9165, 1988). Furthermore, modified prothrombin which is derived from one species may be used in the methods of the invention to assess the platelet activation state of another species. For example, modified prothrombin which is derived from a cow may be used in assessing the platelet activation state of a human.

Various chemical modifications are expected to yield modified prothrombin for use in the present invention. For example, Nakagomi and Ajisaka (Biochem. Int. 22:75, 1990) describe acetyl, succinyl, maleyl, polythylene glycol (PEG), and acetylated-PEG

thrombin, which modifications reduced clotting activities of thrombin. Other chemically modified prothrombins have been described as well (Landaburu and Seegers, Can. J. Biochem. Physiol., 38:613-620;White *et al.*, J. Biol. Chem., 256:1763-1766, 1981 [chemically modified a small number of the lysine residues of human α-thrombin using pyridoxal 5′-phosphate]; Morrison, Int. J. Pept. Prot. Res., 24:377-385, 1984 [nucleophilic reagent (dichlorotriazinylaminofluorescein)]). In a specific embodiment, *infra*, the acetylating agent is sulfo-*N*-succinimidyl acetate.

The acetylation conditions, and the properties of the acylated prothrombin produced that render it applicable to the assay of platelet state, are key features of the preferred embodiments. Thus, acetylation may be done under controlled conditions with the commercially available reagent sulfo-N-succinimidylacetate. Measurement of prothrombin activation by the enzyme factor Xa in the presence of platelets may be done chromogenically using a commercially available chromogenic substrate for thrombin. In addition, control experiments may be performed in which prothrombin cleavage is assayed in the presence of either exogenously added factor Va and anionic phospholipids or artificially activated platelets. Platelets may be artificially activated by many methods including incubation with a calcium ionophore.

Other possible modifications include, but are by no means limited to, site directed mutagenesis of prothrombin, *e.g.*, at the serine residue, so that the generated thrombin is inactivated. Mutated versions of prothrombin comprising deletions of amino acid sequences are within the scope of the invention.

Modified versions of prothrombin, which when catalyzed by prothrombinase yield a modified version of thrombin which does not activate platelets, exist as very rare congenitally mutant proteins and as molecules produced by chemical modification of prothrombin. Landaburu and Seegers (Can. J. Biochem. Physiol., 38:613-620) demonstrated nearly 40 years ago that acetylation of prothrombin produces a protein that can be activated by prothrombinase, but the acetylated thrombin that is generated is inactive on fibrinogen, even though it retains proteolytic activity on small substrates like amino acid or peptide esters and amides. White and colleagues (J. Biol. Chem., 256:1763-1766, 1981) chemically modified a small number of the lysine residues of human α-thrombin using pyridoxal 5′-

phosphate and showed that the modified enzyme is defective not only in fibrinogen cleavage but also in platelet aggregation. Similarly, Morrison (Int. J. Pept. Prot. Res., 24:377-385, 1984) observed that reaction of prothrombin with a nucleophilic reagent (dichlorotriazinyl-aminofluorescein) produced a molecule that was activated normally, but generated a defective thrombin with less than 5% activity on fibrinogen. Two congenital mutants of prothrombin with very similar properties were described over the same period, the prothrombin alleles *Metz* (Rabiet *et al.*, Blood, 63:927-934, 1984) and *Quick I* (Henriksen and Owen, J. Biol. Chem., 262:4664-4669, 1987; Henriksen and Mann, Biochemistry, 27:9160-9165, 1988). Both can be activated by the prothrombinase complex, but the thrombin species produced, just as with chemically modified protein, are defective in their action on both fibrinogen and platelets. Interestingly, a chemical-modification study of another clotting zymogen has shown that this behavior is not unique to prothrombin: acetylation of factor X under mild conditions produces a molecule that is activated normally, but the acetylated factor Xa generated has no activity on two macromolecular substrates that were tested, prothrombin and factor VIII (Morrison *et al.*, Biochim. Biophys. Acta, 884:409-418, 1986; Neuenschwaner and Jesty, Anal. Biochem., 184:347-352, 1990).

Prothrombin can be obtained from any animal source, preferably a mammalian source, and most preferably human. Because of the high degree of prothrombin evolutionary conservation, any orthologue thereof is suitable for use in the present invention. A preferred source of prothrombin, because it is readily available, is bovine prothrombin. Other species include but are not limited to pig, goat, sheep, horse, mouse, rat, rabbit, chicken, turkey, and the like. Prothrombin 00may be obtained from animal sources or can be expressed recombinantly using the nucleic acids of any animal. It can be modified as described above.

Assays for Thrombin Generated from Modified Prothrombin

Any technique known in the art can be used to detect, and, if desired, quantify, thrombin production in an assay of the invention. Examples of such assays include, but are not limited to polyacrylamide gel electrophoresis analysis assays and immunoassays (including Western blotting, ELISA, immunodiffusion, surface plasmin resonance (i.e., Biacore AB), magnetic bead, fluorescence proximity assays, luminescence assays, and the

like) for thrombin. Any such immunoassay would distinguish thrombin from prothrombin, whether by comparison of assay data with a size or apparent molecular weight standard (such as in Western blot analysis), or by using an antibody specific for an epitope present on thrombin and absent on prothrombin (naturally the reverse specificity could also be employed, such that the antibody detects an epitope on prothrombin that is lost during the generation of thrombin, and the presence of thrombin detected by the absence of antibody binding). Immunoassay formats include direct binding assays, indirect binding assays, competitive binding assays, and release assays. The methods of the invention may include the use of tagged prothrombin. The tags to which prothrombin may be fused may allow thrombin, which is produced by prothrombinase catalysis of prothrombin, to be selectively purified from unreacted prothrombin and then quantitated. Furthermore, the tag may itself impart an activity upon the protein with which it is fused so as to allow the quantity of the fusion protein to be evaluated by measurement of said activity. For example, glutathione-S-transferase (GST) fusion proteins may be quantitated by measurement of GST activity. Suitable tags may include polyhistidine, malE or other epitope tags.

Labeled thrombin may be detected by polyacrylamide gel electrophoresis, gel permeation chromatography, western blot analysis or other biochemical techniques capable of distinguishing thrombin from prothrombin.

Preferably, as exemplified *infra*, thrombin is detected by detecting an activity of thrombin, such as amidolytic activity (*i.e.*, the proteolytic activity). In a preferred embodiment, a chromogenic substrate is used. A "chromogenic substrate" means a substrate that forms an optically detectable reaction product when exposed to thrombin. A classical chromogenic substrate is a *p*-nitroanilide-peptide, *e.g.*, tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide. Such a peptide is cleaved by a proteolytic enzyme to cleave the *p*-nitroanilide group, which then acquires a yellow color. Chromogenic substrates are particularly attractive for automated testing. Such an enzyme activity assay necessarily requires a modified thrombin that, although defective for platelet activation, retains catalytic activity.

Still other substrates may fluoresce or luminesce after cleavage by thrombin (termed herein a fluorogenic or luminogenic substrate), and these can be adapted to use in the invention as well.

Biomedical Devices With Thromboembolic Potential

All biomedical devices that either recirculate blood or are inserted into the blood stream have damaging effects on the various blood components. The thromboembolic potential induced by such devices is directly related to the risk of cardioembolic stroke that such devices carry. The blood flows around and/or through such devices. The method described in this disclosure enables the quantitative measurement of the activation state of platelets by a simple and sensitive method, and is particularly useful for assessing the procoagulant properties of various biomedical devices. For example, the initial platelet activation state of a blood sample may be assessed, after exposure of the blood to a biomedical device, the measurement may be repeated. These measurements may be performed on the blood of a patient both before and after exposure to the device. The measurements may also be performed wherein the platelet activation state of blood obtained from blood vessels leading to and from a biomedical device are measured and then compared. A partial, non-limiting, list of biomedical device which may be evaluated with the methods of the present invention includes: prosthetic heart valves, ventricular assist devices (VAD), artificial hearts, heart-lung machines, dialysis machines, intra-aortic balloon angioplasty (PCTA) devices, aortic stents. intra-aortic catheters, endoscopic instruments, prosthetic grafts, and blood-separation and collection systems.

Coagulation - Relation Conditions

The large majority of patients who present with a myocardial infarction, pulmonary embolus, or thrombotic stroke do so with little warning, and the majority of these have no measurable hemostatic abnormalities that are clear causes of the prothrombotic condition. With the few exceptions of specific clotting protein defects that are linked to thromboembolic disease, there is no predictive quantitative test of the hemostasis system that correlates with thrombotic risk. The present invention permits quantitation of an individual's endogenous platelet activation state, and it has the potential for evaluating patients both before and after they suffer thrombotic disease.

For example, patients presenting in a hospital with symptomology that places them at high risk for infarction and/or cardioembolism, e.g., myocardial infarction or stroke,

can be quickly and inexpensively tested. A test result indicating thrombotic potential due to platelet activation would result in prescription of appropriate pharmaceutical therapies.

However, the invention need not be limited only to testing subjects at risk for pathological thrombosis. Subjects preparing for surgery or who have suffered traumatic injury can be tested to ensure that their thrombotic potential is high enough to permit therapeutic clotting. These patients can benefit from pro-coagulation treatments if required.

Kits

Naturally, the components for practice of the invention can be provided in kit form for the convenience of a practitioner. Such a kit can be complete in itself, or can provide unit amounts of reagents for use in conjunction with an automated testing apparatus.

The kit of the invention comprises modified prothrombin, as described above. Alternatively, the kit could comprise prothrombin and a modifying reagent, such as the acetylating agent SNSA, to permit preparation of freshly modified prothrombin, and this is contemplated by the term "modified prothrombin" as used in conjunction with a kit of the invention. As generation of the prothrombinase complex on platelets also requires factor Xa, preferably the kit also includes this component. The kit also comprises an assay for the thrombin generated from the modified prothrombin, *e.g.*, immunoassay reagents, biochemical detection reagents, or thrombin activity assay reagents. In a preferred embodiment, the thrombin assay is an assay for an activity of thrombin. Such an assay can comprise an assay for thrombin amidolytic activity. In a specific embodiment, the kit comprises a chromogenic substrate, or alternatively a fluorogenic or luminogenic substrate.

A kit of the invention may contain a positive control sample of thrombin, *e.g.*, human α-thrombin. The kit may also contain material, such as calcium ionophore, to provide means of fully activating platelets. Such control samples are particularly useful for standardizing the assay and quantitating thrombin production levels.

Components supplied in the kit may be provided in appropriate vials or containers, *e.g.*, plastic or glass vials. The modified prothrombin, assay reagents, and any control reagents can be lyophilized or provided in a ready to use or concentrated liquid form.

The kit can include appropriate label directions for storage, and appropriate instructions for usage.

EXAMPLES

The present invention will be better understood by reference to the following examples, which are provided to illustrate one embodiment of the invention and not by way of limitation.

EXAMPLE 1: Acetylated Prothrombin as a Substrate in the Measurement of Procoagulant Activity of Platelets:Elimination of the Feedback Activation

This example is an evaluation of acetylated prothrombin as a substrate for prothrombinase and an evaluation of the ability of acetylated thrombin to cleave fibrinogen and to activate platelets.

Materials and Methods

Materials. Bovine serum albumin (BSA; fatty acid free, fraction V), calcium ionophore A23187, phosphatidylserine(PS), phosphatidylcholine(PC), and common reagent-grade chemicals were obtained from Sigma Chemical, St Louis, MO. Tricine [N(tris-hydroxymethyl)-methylglycine] and HEPES (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) was from Calbiochem-Behring, San Diego, CA. The chromogenic amide substrate for thrombin was Chromozym-TH (tosyl-Gly-L-Pro-L-Arg-*p*-nitroanilide), from Boehringer Mannheim, IN. BioGel A-15M was obtained from BioRad Laboratories, Richmond, CA. Sulfo-N-succinimidyl acetate (SNSA) obtained from Pierce Chemical, Rockford, IL. Phosphatidylserine and phosphatidylcholine were prepared as a sonicated 30:70 PS:PC (vesicles comprising PS and PC) mixture, at a stock concentration of 2 mg/ml in 0.1 M NaCl 0.05 M Tris/HCl, pH 7.5.

Prothrombin-deficient plasma for clotting assays was prepared. Blood was collected into 1/100 vol 40% sodium citrate and centrifuged at 2500 × *g* for 15 min to yield plasma. One twentieth vol of 1.5 M $BaCl_2$ was added to the plasma on ice, the mixture

stirred for 15 min, and the barium citrate precipitate was removed by centrifugation (15,000 × *g* for 10 min). To remove the excess free Ba^{2+} in the plasma, Na_2SO_4 was added to a concentration of 70 mM and stirred for 10 min on ice. The resulting $BaSO_4$ was removed by centrifugation. Finally, sodium citrate was added to a concentration of 10 mM to mimic the free citrate concentration in ordinary congenitally deficient plasma.

Human factor X was prepared by the method of Morrison and Jesty (Blood, 63:1338-1347, 1984), and human factor Xa by the method of Jesty and Nemerson (Jesty and Nemerson, Meth. Enzymol., 45:95-107, 1976). The concentration of factor Xa was determined by reference to a standard factor Xa that had been assayed by titration with pure bovine antithrombin III. Bovine factor Va was prepared as described by Martin and Jesty (J. Biol. Chem., 270:10468-10474, 1995). Human α-thrombin was prepared by a modification of the method of Fenton *et al.* (J. Biol. Chem., 252:3587-3598, 1977) using cation-exchange chromatography on CM-Sephadex.

Acetylation. Prothrombin (10μM) was dialyzed against 0.1 M $NaHCO_3$ (pH 8.2) in the absence of divalent metal ions, and then acetylated with SNSA at room temperature for time courses up to 90 minutes, using reagent concentrations of 0.1, 0.3, 1, 3, and 10 mM. To perform acetylation, SNSA was dissolved in dry dimethylsulfoxide at a concentration of 100 mM. The acetylation reactions were then started by the addition and mixing of the appropriate amount of this solution to a sample of the prothrombin. The reaction with the reagent was stopped by the addition of 1/10 vol 0.5 M tris-HCl pH 8. The product was assayed using two methods. (1) The clotting activity was measured by a 1-stage prothrombin clotting assay using barium-adsorbed prothrombin-deficient plasma (see above) containing 0.1 mg/ml soybean lecithin and bovine factor Xa (0.1 μg/ml) as activator. The assay was standardized with purified human prothrombin over the range 0.3–150 nM. (2) Acetylated or control prothrombin, 100 nM, was activated by factor Xa in the presence of bovine factor Va and PS:PC, and the rate of thrombin generation determined by chromogenic assay (see ***Assay of prothrombin activation***, below).

Isolation of Platelets. Blood (100 ml) was drawn by venipuncture from a healthy volunteer into 1 ml of 40% trisodium citrate and centrifuged at 400 × *g* for 10 min to obtain platelet-rich plasma. The platelets were separated from the plasma by gel filtration at

room temperature of a 15-ml sample in a 240-ml column of Bio-Gel A-15M, 50-100 mesh, equilibrated in buffer containing 0.1% BSA, 5 mM glucose, 135 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $MgCl_2$, and 10 mM HEPES-NaOH, pH 7.4 (Neuenschwander and Jesty, Blood, 72:1761-1770, 1988). The resulting plasma-free platelets were counted with a Coulter ZM counter (Coulter, Hialeah, FL). Platelets isolated in this way were used within eight hours of their isolation. It should be noted that, while gel filtration is a physically mild method of isolation, no special procedures were used to further reduce the activation of platelets during the procedure.

Assay of prothrombin activation. In these experiments, the extent of prothrombinase catalyzed prothrombin cleavage was measured by quantitation of the proteolytic activity of the thrombin which is produced. Prothrombinase reactions were conducted at 37°C in a volume of 250 µl containing HBS/BSA (0.1% BSA in 0.13 M NaCl / 20 mM Hepes-NaOH pH 7.4). Each reaction contained normal or acetylated prothrombin (100 nM), 50 pM factor Xa, plus either 10 µM PS:PC or platelets (10,000/µl), and 5 mM $CaCl_2$. The reactions were stopped at several time points; at each time point, 20 µl of the reaction mixture were removed into tubes containing 180 µl 10 mM EDTA in HBS/BSA, which stops further prothrombin activation. The diluted samples, 20 µl were then assayed for thrombin activity in microplate wells containing 50 µl 1 mM Chromozym-TH plus 50 µl 50 mM tricine-HCl pH 8.3. As the reaction progresses, the A_{405} nm increases due to cleavage of the Chromozym TH substrate by thrombin. Absorbance at 405 nm was measured at 10 second intervals, and the data were then fitted to obtain an initial slope, ΔA_{405}/min, in each well. Thrombin concentrations in each sample were obtained by comparison of the initial slope to that of a pure preparation of thrombin. The initial rates of thrombin generation in each experiment were generally determined from secondary plots of the course of thrombin generation by linear regression. However, in the case of the most rapid activations, which showed a significant reduction in rate during even a short incubation course, good fits were obtained by assuming the kinetics to be approximately first order with respect to prothrombin concentration (see Figure 1). These data were therefore fitted to a single exponential, $E_t = E_{max}(1 - e^{-kt})$, which on differentiation gives the initial slope as $E_{max}k$.

To this point the assay of platelet procoagulant activity has not been formally standardized. This is particularly important given the variability in the acetylated prothrombin substrate. In the course of this study we have used two checks of the assay, either of which might be developed as a standardization procedure: 1) the use of heavily sonicated platelets (*e.g.* 25 W for 1 minute at 0°C) which provide a source of platelet membrane vesicles which contain *inter alia* randomized PS and PC and factor Va. This has the disadvantage of the significant variation in platelets among individual donors, and the fact that the activation state of the platelet factor V in such a preparation is unknown, 2) and a prothrombinase standard which uses purified factor Va and PS:PC vesicles, but this is less closely related to platelets.

Prothrombin activation with ionophore activated or inactivated platelets. Platelets were incubated for 2 min at 37°C with unmodified or acetylated prothrombin and $CaCl_2$, plus and minus 10 μM A23187. Factor Xa, 1/20 vol, was then added to start prothrombin activation. The final concentrations of each reactant in the activation phase were as follows: 10,000 platelets/μl, 100 nM prothrombin (unmodified or acetylated), 5 mM $CaCl_2$, and 50 pM factor Xa. Samples, 20 μl, were removed at the times shown into 180 μl 10 mM EDTA in HBS/BSA. Samples of each of these dilutions, 20 μl, were assayed for thrombin chromogenic activity. The thrombin concentrations, derived from a standard of pure α-thrombin, represent the thrombin amidolytic level in the prothrombin-activation incubation.

Prothrombin activation with thrombin activated or inactivated platelets. Platelets (10,000 per μl) were incubated for 5 min in the presence factor Va, PS:PC, $CaCl_2$ and unmodified or acetylated prothrombin, as described in ***Prothrombin activation with ionophore activated or inactivated platelets***, plus α-thrombin at concentrations of 0, 10 nM , and 50 nM. Factor Xa was then added to start prothrombin activation. Samples were removed and assayed for thrombin generation as described in ***Prothrombin activation with ionophore activated or inactivated platelets***.

Results

Prothrombin acetylation conditions. Prothrombin preparations were acetylated with various concentrations of SNSA. The (i) activity on platelets, and (ii) the

amidolytic activity on a *p*-nitroanilide peptide substrate of the thrombin product produced by prothrombinase catalysis of these preparations was evaluated. By comparison of the clotting activity and the amidolytic activity of unmodified thrombin and acetylated thrombin which is produced by the prothrombinase reaction, the optimal prothrombin acetylation conditions may be determined. To test the ability of these preparations to activate platelets we performed clotting assays (White *et al.* 1981). In the clotting assays thrombin generation was measured by quantization of thrombin activity on a fibrinogen substrate. Amidolytic activity of acetylated thrombin was tested by activating each preparation of acetylated prothrombin under standard conditions with exogenous complete prothrombinase complex (factors Xa + Va + PS:PC vesicles), and measuring the generation of thrombin in a chromogenic assay.

TABLE 1 shows the results of these assays after acetylation of prothrombin with varying concentrations of SNSA in 0.1 M $NaHCO_3$ (pH 8.2) at room temperature for 30 min. The activity measured by clotting assay is expressed as a fraction of the activity of unmodified prothrombin. The generated chromogenic activity is expressed in terms of the rate of generation of thrombin chromogenic activity, determined by assay of discontinuous timed samples from mixtures containing prothrombinase complex and (modified) prothrombin. It will be noted that all of the acetylated prothrombin preparations, except that made with 10 mM SNSA, generate more amidolytic activity than does normal prothrombin. Such increases in the activity of acetylated clotting proteases on ester and amide substrates have been previously reported (Landaburu and Seegers, *supra*, 1960; White *et al.*, *supra*, 1981; and, Morrison *et al.*, *supra*, 1986). In the range 1-3 mM SNSA (30-min treatment, pH 8.2, room temperature), essentially total inactivation of clotting activity occurs (greater than or equal to 99.8%), while generation of chromogenic activity remains satisfactory in all cases.

TABLE 1 Clotting activity and amidolytic activity of various acetylated thrombin preparations

Concentration of SNSA (mM) used to acetylate prothrombin precursor	Clotting Activity (percent of normal)	Rate of Thrombin Generation (nM/min)
0	100	27.4
0.3	3.75	34.2
1	0.12	33.4
3	< 0.1	34.2
10	< 0.1	16.9

Prothrombin was acetylated with SNSA at pH 8.2 at room temperature for 30 min. Samples were assayed by clotting assay and by chromogenic measurement of the rate of activation by the prothrombinase complex (factor Xa + Va + PS:PC).

However, although acetylation causes no reduction in the rate of activation of prothrombin by factor Xa + Va + PS:PC, when activated platelets are used in place of factor Va + PS:PC there is a large reduction in the activation rate of the acetylated molecule (see below).

Activation of normal prothrombin in the presence of unactivated and activated platelets. The generation of thrombin chromogenic activity from normal prothrombin in the presence of factor Xa plus unactivated and activated platelets, 2×10^7 per ml, is shown in FIGURE 1. A high concentration of calcium ionophore A23187 was used as the platelet activator. The platelet count was chosen to ensure that the platelets are limiting in providing prothrombinase activity; the rate of thrombin generation is a measure of the platelet contribution. Under these conditions activated platelets provide both of the required cofactors: factor Va and anionic phospholipid membrane. In the absence of prior activation of the platelets (FIGURE1, open circles) the generation of thrombin shows a characteristic lag phase, indicating a requirement for feedback activation of the platelets during the incubation. Prior treatment of the platelets with calcium ionophore (FIGURE 1, solid circles) eliminates this lag confirming the requirement for platelet activation.

Activation of acetylated prothrombin in the presence of platelets. FIGURE2 shows the corresponding activation of acetylated prothrombin under the same conditions. This FIGURE indicates a large reduction in the rate of generation of chromogenic activity compared with that in FIGURE 1. This is in direct contrast to the full rate of thrombin generation that is obtained when exogenous factor Va and PS:PC, rather than platelets, are used to activate acetylated prothrombin (TABLE 1). The cause of this is unknown, but, without being bound by a theory, it strongly suggests that the interaction of prothrombin with activated platelets is highly dependent on another site on the molecule that is modified by acetylation. Despite the lower reactivity, the effect of acetylation is clear; in the absence of platelet activation (FIGURE 2, open circles), the generation of thrombin remains linear with time. By comparison with FIGURE 1, it is clear that platelet activation is not occurring during the assay. However, prior platelet activation with calcium ionophore causes a 5-fold increase in the thrombin generation rate (FIGURE 2, solid circles).

Thrombin activation of platelet. To determine if feedback activation of platelets by thrombin was responsible for the increasing, non-linear rate of thrombin generation in the platelet-dependent activation of normal thrombin as observed in FIGURE 1, experiments were performed on platelets pre-treated with α-thrombin (FIGURE 3). In the presence of unactivated platelets, there was a lag phase (open circles). However, in the presence of platelets activated with 10 nM α-thrombin, the lag phase in thrombin generation is substantially shortened, but not quite abolished (solid circles); with 50 nM α-thrombin (open square), no lag phase is evident. The thrombin activities at zero time in FIGURE 3 are due to the thrombin used to pre-activate the platelets in each case. It should also be noted that the final rates of thrombin generation in each experiment are nearly identical, showing that the same prothrombinase activity is obtained in each, whether activation by thrombin occurs *in situ* during the incubation, or by exogenous treatment with enzyme.

Thrombin-activated platelets on acetylated prothrombin. FIGURE 4 essentially mimics the use of the assay in measurements of platelet procoagulant activity, showing the activation of acetylated prothrombin in the presence of untreated and thrombin-treated platelets. The rate of thrombin production increases proportionally to the quantity of α-thrombin used to activate the platelets. The open circles represent thrombin production in

the presence of unactivated platelets, closed circles represent that of platelets activated with 10 nM α-thrombin, open squares represent that of 50 nM α-thrombin. As before, the reduction in thrombin generation rate when acetylated prothrombin is activated with platelet-bound prothrombinase is clear. Nonetheless, activation remains easily measurable, and the rate of thrombin generation is linear. Acetylation of prothrombin indeed blocks the feedback activation of platelets during these assays, allowing a quantitative measurement of procoagulant activity of the platelets.

Discussion

The method devised herein is similar to two existing methods (Folie and McIntire, *supra*, 1989). The prothrombinase assay of the procoagulant state of both erythrocytes and platelets is well described (Bevers *et al.*, *supra*, 1991; Martin and Jesty, *supra*, 1995; Schroit and Zwaal, Biochim. Biophys. Acta, 1071:313-329, 1991). Assays of erythrocyte prothrombinase activity, exogenous factor Va is provided in the assay and the prothrombinase activity corresponds with exposure to anionic phospholipid. In the measurement of platelet activation, both factor Va and anionic phospholipid can be provided by the platelets, so factor V need not be exogenously provided. (Slack *et al.*, *supra*, 1993) The use of unmodified prothrombin in platelet assays is problematic because small amounts of thrombin produced in the early phases of the assay feed back and further stimulate the platelets. For a more reliable assay, a modified prothrombin is needed that (i) is activated at normal or near-normal rates by the prothrombinase complex, and (ii) produces thrombin that cannot activate platelets. The use of acetylated prothrombin for this purpose is similar to a modification of the clotting zymogen factor X, in which acetylation blocked the proteolytic activity of generated factor Xa (Morrison *et al.*, *supra*, 1986). This enabled studies of the activity of unactivated factor VIII in factor X activation (Neuenschwander and Jesty, *supra*, 1995).

Without being bound by theory, it seems likely that acetylation under the conditions used in these studies modifies a binding site in the thrombin domain of prothrombin that is distant from the enzyme's active site (an exosite), but which is required for enzymic activity on macromolecular substrates such as fibrinogen and the platelet

thrombin receptor. In contrast, judging by the near-normal rates of generation of thrombin amidolytic activity on small peptide substrates, acetylation does not appear to significantly alter the activation of prothrombin by factor Xa; i.e. the interactions of acetylated prothrombin with purified components of the prothrombinase complex–factor Xa, factor Va, and anionic phospholipid–appear to be essentially normal.

We observed an unexpected effect of prothrombin acetylation; whereas acetylated prothrombin is activated normally in the presence of purified phospholipid and factor Va, its activation rate is significantly reduced when activated platelets are the source of the prothrombinase complex. This suggests that acetylation may also affect a site through which prothrombin interacts with activated platelets. This site may be separate from the site that is responsible for the reduction of thrombin activity on macromolecular substrates. It is known that acetylation of bovine prothrombin modifies three key residues in the fragment-1 (NH_2-terminal) region of the molecule: specifically, the amino terminal alanine, and two particularly reactive residues in the fragment-1 kringle: His-96 and the carbohydrate-linked Asn-101 (Welsch and Nelsestuen, Biochemistry, 27:4946-4952, 1988; Welsch and Nelsestuen, Biochemistry, 27:4946-4952, 1988; Welsch and Nelsestuen, Biochemistry, 27:7513-7519, 1988). It is possible that one or more of these residues are modified under our acetylation conditions, and are involved in a platelet-specific interaction. Furthermore, it is possible that the major platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) plays a role in the activation of prothrombin (Reverter *et al.*, J. Clin. Invest., 98:863-874, 1996; Byzova and Plow, J. Biol. Chem., 272:27183-27188, 1997) and may interact with an Arg-Gly-Asp (RGD) sequence (residues 560-562, human prothrombin precursor numbering) in the still latent catalytic domain of prothrombin. This RGD-sequence is immediately N-terminal to Ser-568, which is the active-site serine of thrombin. In the active enzyme, the Asp residue of the RGD sequence provides the negative charge at the bottom of the trypsin-family S1 binding pocket. Immediately N-terminal to the RGD sequence is a Lys residue, Lys-559. Without being bound by theory, modification of this residue might cause the defective interaction of acetylated prothrombin with platelets.

The method described in this report enables the quantitative measurement of the activation state of platelets by a simple and sensitive method. This method may be used

to measure platelets derived from any source. As mentioned in the Introduction, one area in which it will be particularly useful is the study of the effects of biomedical devices such as pumps and prosthetic valves on platelets in flow experiments *in vitro*. The activation time of platelets taken directly from the body of a patient may also be evaluated using the methods described herein.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all values are approximate, and are provided for description.

Various patents, patent applications, and publications are cited herein, the disclosures of which are incorporated by reference in their entireties.